ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2007, p. 941–945 Vol. 51, No. 3
0066-4804/07/$08.00+0 doi:10.1128/AAC.00880-06

Caspofungin in Combination with Amphotericin B against *Candida parapsilosis*▽

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Received 17 July 2006/Returned for modification 28 November 2006/Accepted 30 November 2006

Candida parapsilosis **has emerged as an important nosocomial pathogen. In the present study, a checkerboard broth microdilution method was performed to investigate the in vitro activities of caspofungin (CAS) in combination with amphotericin B (AMB) against three clinical isolates of *C. parapsilosis*. Although there was a significant reduction of the MIC of one or both drugs used in combination, an indifferent interaction (fractional inhibitory concentration index greater than 0.50 and less than or equal to 4.0) was observed in 100% of cases. This finding was confirmed by killing curve studies. By a disk diffusion assay, the halo diameters produced by antifungal agents in combination were often significantly greater than those produced by each drug alone. Antagonism was never observed. In a murine model of systemic candidiasis, CAS at either 0.25 or 1 mg/kg/day combined with AMB at 1 mg/kg/day was significantly more effective than each single drug at reducing the colony counts in kidneys. Higher doses of the echinocandin (i.e., 5 and 10 mg/kg/day) combined with the polyene did not show any advantage over CAS alone. Overall, our study showed a positive interaction of CAS and AMB against *C. parapsilosis*.**

The frequency of invasive mycoses due to opportunistic fungal pathogens has increased dramatically over the past two decades, and now *Candida* spp. ranks as the fourth most common cause of nosocomial bloodstream infections (20). Although *Candida albicans* is the organism most often associated with serious fungal infections, other *Candida* spp. have emerged as clinically important pathogens associated with opportunistic infections (17, 20).

Candida parapsilosis is the second most frequent yeast species isolated from normally sterile body sites in North America, Europe, and Latin America (12, 20). Moreover, studies conducted in the United States showed that *C. parapsilosis* is the most common non-*C. albicans Candida* spp. in pediatric patients (17, 18, 23).

Caspofungin (CAS) is an echinocandin antifungal agent that has potent activity against many fungal species, including *Candida* spp. (13, 20, 22). Clinical studies have shown that CAS is at least as active as amphotericin B and fluconazole in the treatment of invasive candidiasis (13, 20).

Amphotericin B (AMB) targets fungal ergosterol, the main component of the fungal cell membrane, while CAS inhibits the synthesis of the fungal cell wall by blocking β-1,3-D-glucan (6, 7). Its innovative mechanism of action makes this drug a suitable candidate for antifungal combination therapy.

Although CAS MICs for *C. parapsilosis* can be higher than those seen for *C. albicans*, the echinocandin is generally effective in infections caused by this yeast species (1, 4, 6–8, 13, 20, 22). Similarly, amphotericin B is active in vitro and in vivo against *C. parapsilosis* (20).

In this study, we hypothesized that the combination of CAS and AMB could be advantageous over each monotherapy against *C. parapsilosis*. To investigate this interaction, we applied in vitro methods and an experimental mouse model of systemic infection.

MATERIALS AND METHODS

Isolates. Two clinical isolates of *C. parapsilosis* (no. 2 and no. 3) and *C. parapsilosis* ATCC 22019 were used in this study. Both clinical isolates were recovered from blood. Yeast isolates were identified at the species level by conventional morphological and biochemical methods and stored at −70°C in 10% glycerol. Before the initiation of the study, yeast isolates were subcultured on antimicrobial agent-free medium to ensure viability and purity.

Drugs. For both in vitro and in vivo studies, stock solutions of CAS (Merck Sharp & Dohme Ltd., Hoddesdon, United Kingdom) were prepared in distilled water. For in vitro studies, stock solutions of AMB (Sigma Chemical, Milan, Italy) were prepared in dimethyl sulfoxide (Sigma). Further dilutions were prepared in the test medium. For in vivo studies, stock solutions of AMB (Fungizone; Bristol-Myers Squibb S.p.A., Latina, Italy) were prepared in sterile distilled water.

In vitro studies. (i) Microdilution method. Drug activity was assessed by a checkerboard method derived from the standardized procedure established by the CLSI for broth microdilution antifungal susceptibility testing (14). Briefly, testing was performed in RPMI 1640 medium (Sigma) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS; Gibco Laboratories, Milan, Italy) buffer. Volumes of 50 μl of each drug at a concentration of four times the targeted final concentration were dispensed in the wells of 96-well microtiter plates (Falcon 3072; Becton Dickinson). The final concentrations of the antifungal agents ranged from 0.03 to 2.0 μg/ml for AMB and from 0.03 to 64 μg/ml for CAS. Yeast inocula (100 μl), prepared spectrophotometrically and further diluted to obtain concentrations ranging from 1.0×10^3 to 5.0×10^3 CFU/ml (2× inoculum), were added to each well of the microdilution trays. The trays were incubated in air at 35°C and read at 24 and 48 h. Readings were performed spectrophotometrically at an optical density at 490 nm (OD_{490}) with an automatic plate reader (ELx800; Biotek). Two MIC endpoints were considered: the first concentration of the antifungal agent tested alone or in combination at which the turbidity in the well was either 50% (50% inhibitory concentration

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▽ Published ahead of print on 11 December 2006.

TABLE 1. In vitro activity of caspofungin and amphotericin B, alone and in combination, by the broth dilution assay against three *Candida parapsilosis* isolates[a]

Reading parameter	Drug(s)	Geometric mean (range) MIC (μg/ml) at time (h):	
		24	48
IC_{50}	CAS	0.79 (0.5–1.0)	1.17 (0.5–4.0)
	AMB	0.16 (0.125–0.25)	0.18 (0.06–0.5)
	CAS-AMB	0.016[b]/0.07[c] (0.007–0.5/0.06–0.125)	0.02[b]/0.09[c] (0.007–0.5/0.06–0.25)
IC_{90}	CAS	1.36 (0.5–4.0)	2.33 (1.0–4.0)
	AMB	0.34 (0.125–1.0)	0.68 (0.125–1.0)
	CAS-AMB	0.06[b]/0.13[c] (0.007–1.0/0.03–0.25)	0.12[b]/0.25[c] (0.007–1.0/0.03–0.5)

[a] Each isolate was tested in quintuplicate.
[b] $P < 0.05$ versus CAS alone.
[c] $P < 0.05$ versus AMB alone.

TABLE 2. In vitro activity of caspofungin and amphotericin B, alone and in combination, by disk diffusion assay against three *Candida parapsilosis* isolates[a]

Reading time (h)	Drug[b]	Halo diam (mm [mean ± SD]) with treatment[b]:			
		AMB 0	AMB 0.1	AMB 1	AMB 10
24	CAS 0	Not detectable	6.7 ± 1.4	11.1 ± 1.8	15.2 ± 2.0
	CAS 1	6.2 ± 0.2	8.1 ± 2.1[c]	13.4 ± 3.1[c]	16.4 ± 3.2[c]
	CAS 10	13.3 ± 2.3	13.4 ± 0.9[d]	16.3 ± 2.2[c,d]	17.3 ± 2.7[c]
	CAS 100	21.1 ± 3.7	19.8 ± 1.7[d]	22.0 ± 2.1[d]	22.1 ± 1.9[d]
48	CAS 0	Not detectable	6.4 ± 1.3	10.6 ± 0.8	13.7 ± 1.5
	CAS 1	6.1 ± 0.1	6.2 ± 0.7	12.8 ± 1.4[c,d]	15.4 ± 1.6[c,d]
	CAS 10	13.2 ± 1.7	13.0 ± 0.7[d]	15.2 ± 0.8[c,d]	15.8 ± 0.8[c,d]
	CAS 100	21.2 ± 2.9	20.1 ± 2.8[d]	21.8 ± 0.8[d]	22.3 ± 1.6[d]

[a] Each isolate was tested in triplicate.
[b] CAS, caspofungin at 1 μg (CAS 1), 10 μg (CAS 10), and 100 μg (CAS 100); AMB, amphotericin B at 0.1 μg (AMB 0.1), 1 μg (AMB 1), and 10 μg (AMB 10).
[c] $P < 0.05$ versus CAS alone.
[d] $P < 0.05$ versus AMB alone.

[IC_{50}]) or 90% (IC_{90}) less than that in the control well (15). Both on-scale and off-scale results were included in the analysis. The high off-scale MICs were converted to the next highest concentration, while the low off-scale MICs were left unchanged. Drug interactions were classified as synergistic, indifferent, or antagonistic on the basis of the fractional inhibitory concentration (FIC) index. The interaction was defined as synergistic if the FIC index was less than or equal to 0.50, indifferent if the FIC index was greater than 0.50 and less than or equal to 4.0, and antagonistic if the FIC index was greater than 4.0 (11). Experiments were conducted in quintuplicate.

(ii) Disk diffusion. Disk diffusion was performed in RPMI 1640 supplemented with 2% glucose and 2% agar buffered with MOPS. Briefly, isolates were inoculated into liquid yeast peptone dextrose (2% glucose, 2% Bacto Peptone, 1% yeast extract; Difco Laboratories) and grown overnight at 35°C. The cells were then pelleted, washed three times with distilled water, and counted with a hemocytometer. The agar surface was inoculated in three directions by using a swab moistened in an inoculum suspension that was adjusted to a 0.5 McFarland standard (approximately 1×10^6 to 5×10^6 CFU/ml). The drugs and the solvent control were pipetted onto 6-mm-diameter BBL disks (Becton Dickinson & Co.). Disks were embedded with 10 μl of either drug alone or drugs in combination. CAS was used at concentrations of 1, 10, and 100 μg; AMB was used at concentrations of 0.1, 1, and 10 μg. After the disks had dried, they were placed onto inoculated agar plates. The plates were incubated at 35°C, and inhibition zone diameters were measured at 24 and 48 h (3). Each disk diffusion assay was performed in triplicate, and mean diameters were reported.

(iii) Time-kill studies. Time-kill experiments were performed with *C. parapsilosis* strain no. 3. Yeast cells from a 24-h growth plate were suspended in 10 ml of sterile distilled water, and the turbidity was adjusted to a 0.5 McFarland standard by spectrophotometric methods. One milliliter of the adjusted fungal suspension was added to 9 ml of either RPMI 1640 medium buffered with MOPS buffer plus an appropriate amount of each drug alone or in combination. Drugs, alone and in combination, were used at 1× the MIC (IC_{90}; CAS, 4 μg/ml; AMB, 1 μg/ml) and 8× the MIC (CAS, 32 μg/ml; AMB, 8 μg/ml) obtained by the broth dilution method. Test solutions were placed on a shaker and incubated at 35°C. At 0, 2, 6, and 24 h following the introduction of the test isolate into the system, 100-μl aliquots were removed from each test solution. After serial 10-fold dilution, a 50-μl aliquot from each dilution was streaked in triplicate onto Sabouraud dextrose agar plates for colony count determination. Following incubation at 35°C for 48 h, the number of CFU on each plate was determined. The limit of detection was 20 CFU/ml. Fungicidal activity was considered to have been achieved when the number of CFU per milliliter was <99.9% of the initial inoculum size. Synergy was defined as a ≥100-fold increase in killing compared with that achieved with the most active single agent, while antagonism was defined as a ≥100-fold decrease in killing compared with that achieved with the most active single agent. If a <100-fold change from the effect of the most active single drug was observed, the interaction was considered indifferent (11, 19). Experiments were conducted in triplicate.

In vivo studies. CD1 male mice (Charles River, Calco, Italy) weighing 25 g were rendered neutropenic by intraperitoneal administration of cyclophosphamide 200 mg/kg of body weight/day on days −4, +1, and + 4 postinfection. They were infected intravenously with *C. parapsilosis* strain no. 3 given in a 0.2-ml volume. Both drugs were administered intraperitoneally in a 0.2-ml volume. The mice were challenged with 3.5×10^8 CFU/mice, treated for 4 consecutive days starting 24 h postchallenge with CAS at 0.25, 1, 5, and 10 mg/kg/day (CAS 0.25, CAS 1, CAS 5, and CAS 10), with AMB at 1 mg/kg/day (AMB 1), or with their respective combinations (COMBO 0.25, COMBO 1, COMBO 5, and COMBO 10). Tissue burden studies were performed on day 5 postinfection. Drug efficacy was assessed by determining the number of CFU per kidney pair. Briefly, the mice were sacrificed, the kidneys were aseptically removed and homogenized, and diluted or undiluted aliquots, including the entire organ, were grown in cultures on Sabouraud dextrose agar for colony count determination. There were 8 animals in each group. Animal experiments were conducted with the approval of University of Ancona Ethics Committee.

Statistical analysis. The in vitro results were analyzed by either Mann-Whitney U test or Student's *t* test considering a *P* value of <0.05 significant. The Mann-Whitney U test was performed to compare tissue burden counts. Due to multiple comparisons, a *P* value of <0.016 was considered statistically significant.

RESULTS

Susceptibility results of the three *C. parapsilosis* isolates are reported in Table 1. At 24 h, the CAS IC_{50} and IC_{90} ranged from 0.5 to 1.0 and from 0.5 to 4.0 μg/ml, respectively. At the same interval, the AMB IC_{50} and IC_{90} ranged from 0.125 to 0.25 and from 0.125 to 1.0 μg/ml, respectively. The combination therapy yielded a significant reduction in the IC_{50} and IC_{90} for both CAS and AMB. Similarly, the checkerboard assay evaluated at 48 h showed that the combination of CAS with AMB significantly decreased the IC_{50} and IC_{90} values with respect to each single drug. However, according to our definition, FIC indexes yielded 100% indifferent interactions either at 24 h or 48 h (FIC indexes ranging from 0.51 to 1.0). Antagonism was never observed.

To further characterize the effects of combination therapy against *C. parapsilosis*, we used a disk diffusion assay. The results are reported in Table 2. In general, at 24 h, the halo diameters produced by different concentrations of combination therapies never exceeded the largest inhibition zone obtained with either CAS or AMB alone. However, CAS at 10 μg combined with AMB at 1 μg yielded halo diameters superior to those obtained by each drug alone ($P < 0.05$). At 48 h, CAS at 1 or 10 μg combined with AMB at either 1 μg or 10 μg produced inhibition zones greater than those of each single drug ($P < 0.05$). Again, antagonism was never observed; in fact, the halo diameters of each drug combination were never smaller than those produced by each drug alone.

The results of killing experiments conducted with *C. para-*

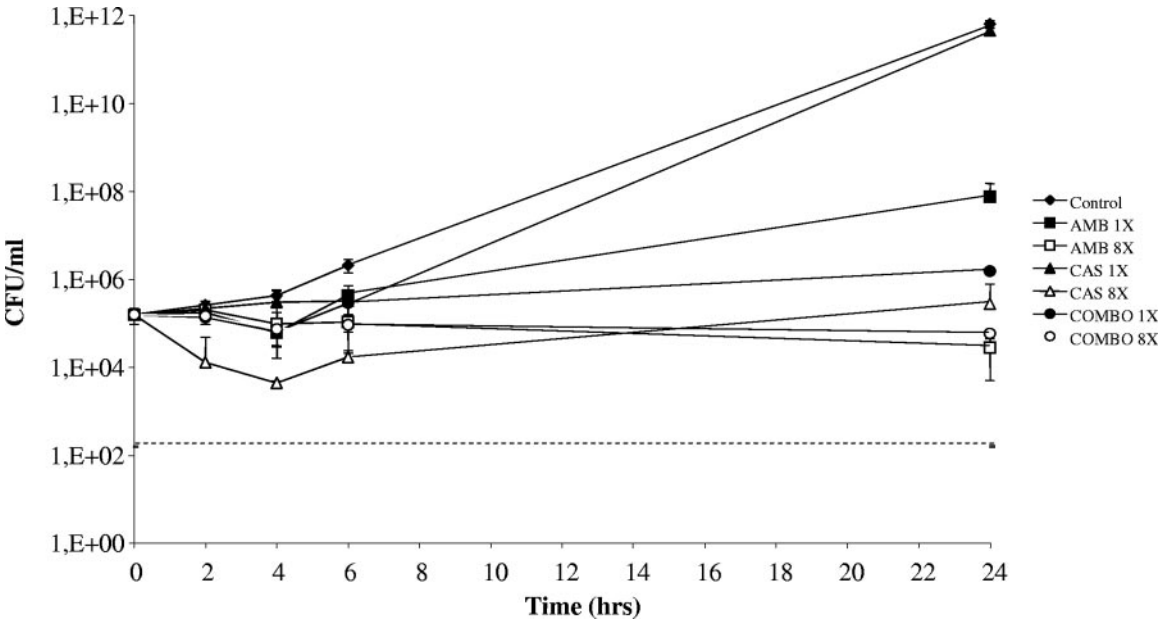


FIG. 1. Time-kill studies conducted with *C. parapsilosis* strain no. 3. AMB 1X, 0.5 μg/ml; AMB 8X, 4 μg/ml; CAS 1X, 4 μg/ml; CAS 8X, 32 μg/ml; COMBO 1X, 0.5 μg/ml AMB plus 4 μg/ml CAS; COMBO 8X, 4 μg/ml AMB plus 32 μg/ml CAS. The dotted lines represent a >99.9% growth reduction compared with the initial inoculum size (fungicidal effect). The limit of detection was 20 CFU/ml. Each datum point represents the mean ± standard deviation of results from three independent experiments.

psilosis strain no. 3 are reported in Fig. 1. Both CAS and AMB showed a dose-dependent activity. CAS used at 1× MIC did not exhibit any antifungal activity (growth at 24 h similar to that of the control), while the echinocandin showed a fungistatic effect at 8× the MIC. Similarly, AMB at 1× the MIC did not exert any antifungal activity, while the polyene yielded a reduction of 0.8 $\log_{10}$ with respect to the initial inoculum at 8× the MIC. Although COMBO 1 was more effective than each drug alone, it yielded only 1.7 $\log_{10}$ growth reduction with respect to AMB alone. COMBO 8 did not show any additional reduction compared to AMB alone. Therefore, combination therapy yielded indifferent interactions regardless of the drug concentrations. Finally, a fungicidal activity was never reached.

To investigate this interaction in vivo, neutropenic CD1 mice were infected intravenously with *C. parapsilosis* strain no. 3 and treated with several therapeutic regimens, including a scheme of combination therapy. The results are reported in Fig. 2. Amphotericin B was effective at reducing the fungal burden against the controls ($P = 0.005$). All CAS doses, with the exception of CAS 0.25, were effective at reducing the counts with respect to the controls ($P = 0.001$). Both COMBO 0.25 and COMBO 1 significantly reduced the tissue burden counts with respect to each single therapy (P ranging from 0.001 to 0.002). COMBO 5 and COMBO 10 were both significantly more effective than AMB alone ($P = 0.001$ and 0.004) but not more effective than the echinocandin given as a single drug.

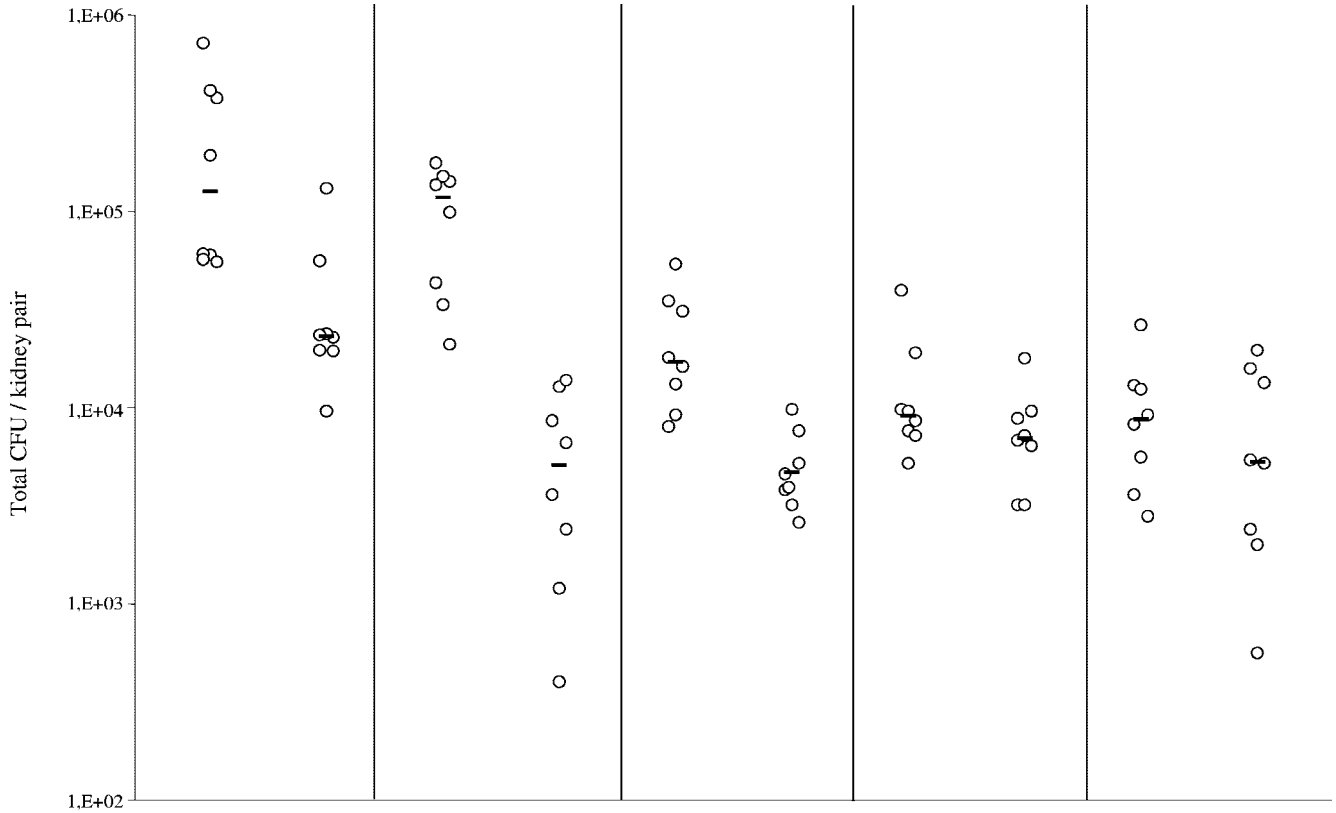
DISCUSSION

In this study, we analyzed the in vitro and in vivo interactions of CAS and AMB against *C. parapsilosis*. To our knowledge, this is the first study addressing the relationship between these two drugs versus this important opportunistic pathogen. Our data are encouraging, since we did not observe any negative effect. The lack of antagonism was documented in vitro and confirmed in vivo.

In vitro interactions were explored by using different methods, including the classical checkerboard dilution methodology, a disk diffusion assay, and the killing curve assay. Although the first method did not support a synergistic interaction, we observed a significant MIC reduction of both drugs upon the combination. Killing curves confirmed these findings. In these experiments, the best interaction was noted when both drugs were combined at 1 rather than at 8 times the MIC. Similarly, the effects observed by the halo assay were influenced by the drug doses utilized in the combination. In particular, only AMB at 1 and 10 μg combined with similar doses of CAS was significantly more effective than each single drug. On the contrary, when the polyene was added to CAS at 100 μg, the combination approach was not superior to the echinocandin alone.

It is interesting that a similar phenomenon was also observed in vivo. Actually, CAS given at 0.25 and 1 mg/kg/day combined with AMB at 1 mg/kg/day was significantly more effective than each single drug at reducing the colony counts in the kidney, while higher doses of the compound (i.e., CAS at 5 and 10 mg/kg/day) added to the polyene did not show any advantage over CAS alone. This can be due to the fact that CAS given as a single drug did not show a significant improvement in the clearance of fungal burden with dose escalation above 1 mg/kg/day. These results are similar to those recently reported by others (1, 5, 9). Altogether, these data indicate that, to obtain a beneficial effect of this combination approach against *C. parapsilosis*, an adequate ratio of drug concentrations has to be achieved.

Literature data addressing the relationships between echinocandins and polyenes against *Candida* spp. are limited. Early



	C	AMB 1	CAS 0.25	COMBO 0.25	CAS 1	COMBO 1	CAS 5	COMBO 5	CAS 10	COMBO 10
Any treatment *vs* C	-	0.005*	0.208	0.001*	0.001*	0.001*	0.001*	0.001*	0.001*	0.001*
Combo *vs* AMB				0.001*		0.001*		0.001*		0.004*
Combo *vs* CAS				0.001*		0.002*		0.141		0.462

FIG. 2. Kidney tissue burden of neutropenic CD1 mice. The mice were infected intravenously with 3.5×10^8 CFU/mice of *C. parapsilosis* strain no. 3. Animals were treated daily for four consecutive days with AMB at 1 mg/kg/day (AMB 1), with CAS at 0.25, 1, 5, or 10 mg/kg/day (CAS 0.25, CAS 1, CAS 5, CAS10), or with their respective combinations (COMBO 0.25, COMBO 1, COMBO 5, and COMBO10). Tissue burden experiments were performed on day 5 postinfection. The bars represent the medians. C, control; *, $P < 0.016$.

experience with cilofungin and AMB in mice with disseminated candidiasis due to *C. albicans* indicated improved survival and reduced tissue burden relative to the results seen with either agent alone (21). More recently, Hossain et al., evaluated in vitro and in vivo efficacies of CAS plus AMB against an azole-resistant strain of *C. albicans* (10). While they found an indifferent interaction in vitro by the checkerboard dilution method, the combination approach was the only treatment that resulted in significant reduction in kidney CFU counts. Recent data showed that either CAS or micafungin administered in combination with AMB were the only therapeutic approaches yielding organ sterilization in murine candidemia models due to *Candida glabrata* (2, 16). In the present study, we expanded the knowledge of this interaction in infections due to *C. parapsilosis*. Unlike previous reports on *C. glabrata*, we did not observe organ sterilization upon combination therapy. This finding can be due to the fact that, in the present study, AMB was utilized only at 1 mg/kg/day, while in previous studies with *C. glabrata*, kidney sterilization was reached when CAS was associated either with AMB at 3 mg/kg/day or with liposomal AMB at 7.5 mg/kg/day (2, 16).

The positive interaction between an echinocandin compound and a polyene can be explained by the fact that both drug families possess unique mechanisms of action. It can be postulated that the candins, which inhibit cell wall synthesis, may enhance the activity of AMB by increasing the rate or degree of their access to the cell membrane.

Although we found a positive interaction between CAS and AMB versus *C. parapsilosis*, an extrapolation of these results into the clinical practice should be done with caution. Actually, literature data showed that AMB, fluconazole, and CAS given as monotherapies are already effective in systemic infections due to this species of *Candida* (20). Thus, this combination approach should be reserved for special clinical settings, such as severe immunocompromised patients with systemic infections, patients with endocarditis, or patients with recurrent infections despite an appropriate antifungal therapy.

In conclusion, combinations of CAS and AMB appear to produce enhanced activity against *C. parapsilosis* both in vitro and in vivo. Additional studies involving several isolates and multiple dosing regimens are warranted to further elucidate the potential benefit of this combination approach in infections due to this common species of *Candida*.

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